Research Thesis: Aptamer von Willebrand Factor Inhibition in Small and Large Stroke Models

Presented in partial fulfillment of the requirements for graduation *with Research Distinction* in *Neuroscience* in the undergraduate colleges of The Ohio State University

Ian A. Mandybur

The Ohio State University
May 2019

Project Advisor: Dr. Shahid Nimjee, MD, PhD
Associate Professor, Department of Neurological Surgery
Wexner Medical Center at The Ohio State University

TABLE OF CONTENTS

I. ABSTRACT:

Occlusive arterial thrombosis leading to stroke and myocardial infarction contribute to ~13 million global deaths annually. In 1995, Intravenous recombinant tissue plasminogen activator (rTPA) represented a breakthrough in stroke therapy, reducing morbidity and improving functional outcomes.[2] However, it was found that patients treated with rTPA routinely result in inadequate recanalization in the setting of large vessel occlusion, experienced restenosis, and suffered from symptomatic intracranial hemorrhage (ICH).[2] Moreover, given that patients must receive rTPA within 3 to 4.5 hours from symptom onset and not have one of the 18-point exclusion criteria, only ~6% of patients received rTPA of the 690,000 ischemic stroke incidents that occurred in 2017.[1-3] Given all of this**, a change from the current stroke therapeutics must be investigated to improve safety and efficacy**.

Aptamers are single-stranded oligonucleotides (RNA and DNA) that can bind to and inhibit targeted proteins. Unlike rTPA and parenteral anti-platelet drugs, aptamers can be reversed with antidote oligonucleotides, that bind to the aptamer via Watson-Crick base-pairing without perturbing platelet reactivity. In this study, we propose a paradigm shift from the current stroke therapeutics to a new target resulting in a more effective, safer, and readily reversible anti-thrombotic agent. Our laboratory has developed an RNA aptamer therapeutic (DTRI-031) which directly inhibits von Willebrand Factor (VWF)-mediated platelet adhesion and aggregation. Along with a matched antidote DTRI-025 to control its activity, we demonstrate that DTRI-031 can lyse stabilized clots in a safer and more effective fashion than rTPA. Furthermore, the information included in this project can provide considerable insight into the use of antidote molecules for safer therapeutic drugs.

II. INTRODUCTION

A. Historical Background

According to data from the National Stroke Association, cerebrovascular disease (stroke) is the fifth leading cause of death in the United States with more than 800,000 Americans experiencing a new or recurrent stroke each year. Every 40 seconds a patient suffers a stroke and every 4 minutes a stroke is fatal.[1] Stroke can be classified into two separate categories, hemorrhagic or ischemic. Hemorrhagic stroke is caused by the rupture of blood vessels within the brain, whereas ischemic stroke is caused by a blockage within a vessel preventing oxygen and nutrients from reaching the brain. Of all strokes presented clinically, 87% have been identified as ischemic. Intravenous recombinant tissue plasminogen activator (rTPA) has been the mainstay of therapy for ischemic stroke treatment since 1995. While the therapeutic window was extended to 4.5 hours from symptom onset, rTPA therapy remains limited to 5-10% of stroke patients with a 6.4% hemorrhagic rate and dismal recanalization rate.[1,2]

An additional class of drugs have been studied in stroke targeting *GPIIb*/*IIIa*, one of the key integrins involved in platelet aggregation and, therefore, blood clot formation; Abciximab, Eptifibatide and Tirofiban. All 3 drugs are used in treating cardiovascular disease but have resulted in poor outcomes in the brain related to uncontrolled hemorrhage.

More recently, clinical trials have demonstrated endovascular thrombectomy with stent retrievers resulted in superior neurological outcomes compared to rTPA in patients who present with large vessel occlusion (LVO).[3,4] This is defined as a clot in the internal carotid artery (ICA) segment of the brain, known as a the supraglenoid ICA, or a clot in the first segment of the middle cerebral artery (M1) or anterior cerebral artery (A1).

In patients who develop a significant stroke with edema, a hemicraniectomy can be performed to reduce mortality and modestly improve neurological outcome.[6] Despite all the

listed acute and subacute therapies, stroke remains the single largest cause of morbidity and mortality in the world today.[3-5] Therefore, an urgent need exists to improve the morbidity and mortality of ischemic stroke.

B. Hemostasis and Coagulation

Understanding blood clot formation is critical to identifying an optimal target for stroke treatment. Normal hemostasis, considered the first stage of wound healing, is the result of well-regulated steps to prevent hemorrhage and maintain blood in a clot-free state in vessels. After vascular injury, coagulation is initiated by rapidly localizing circulating platelets to the site of injury. The exposed extracellular matrix of the injured vessel creates a highly thrombogenic environment which causes circulating platelets to become activated and adhere to the damaged endothelium. Specifically, the subendothelial von Willebrand Factor (VWF) that is exposed as a result of vascular injury binds to the platelet expressed GP Ib-IX-V, resulting in platelet activation and adhesion to the damaged endothelial surface.[7] In response to platelet activation, secretory granules are released, inducing aggregation of additional platelets to form a hemostatic plug.[7,8] Altogether, the interaction between coagulation factors in platelets is crucial to the formation of a thrombus.

Coagulation can be understood from two different mechanisms of hemostasis: the first is a clotting cascade which demonstrates clotting as two separate pathways consisting of sequential steps of activation to form a common pathway; the second is a cell-based model of coagulation which centers on coagulation occurring on the platelet surface.[7] While the classical model of the coagulation cascade correlates well with laboratory tests and describes clotting neatly with an intrinsic and extrinsic pathway, it fails to properly describe coagulation in vivo. The cell-based model of coagulation illustrates the formation of a blood clot in three overlapping phases: initiation, amplification, and propagation (**Figure 1**).[7]

C. von Willebrand Factor

Von Willebrand Factor (VWF) is a large multimeric glycoprotein involved in the seminal event of platelet plug formation.[9,10] When vascular injury occurs, subendothelial collagen becomes exposed to the circulating blood, allowing VWF to serve as a "bridge" between the exposed collagen and platelets as well as between platelets themselves.[9] In particular, VWF interacts with glycoprotein Ibα on platelets to induce adhesion to the vessel wall. Following this, glycoprotein IIb/IIIa becomes activated, binds to fibrinogen, and results in aggregation and thrombus formation (**Figure 1**).

Numerous studies have indicated that the risk of myocardial infarction and stroke correlate with VWF levels among patients at risk.[17,18] Inhibitors of VWF have shown promise to limiting thrombosis,[19-22] however their effect on hemostasis can lead to significant bleeding in the surgical setting.[21-23] In response to this, patients in need of platelet inhibition in the perioperative setting would benefit from a rapidly controllable VWF inhibitor. Furthermore, Von Willebrand Disease (VWD), resulting from a qualitative and/or quantitative reduction in VWF, presents with menorrhagia in women or bleeding in patients undergoing dental procedures, but rarely results in spontaneous hemorrhage.[11] Most importantly, patients with VWD are protected from cerebrovascular and cardiovascular events; therefore VWF, often overlooked, is an attractive target for disorders resulting from arterial thrombosis.[12]

D. Aptamers

Aptamers are a class of DNA or RNA ligands that act like protein inhibitors much like monoclonal antibodies and small molecules.[13] They function by folding into 3-D structures, binding to and inhibiting the function of target proteins with high affinity and specificity. Aptamers are isolated by systematic evolution of ligands by exponential enrichment (SELEX) (**Figure 2**). They can undergo extensive pharmacological revisions to tailor each drug for a specific clinical need.[13]

While most anti-thrombotic therapeutics routinely come with the risk of hemorrhage due to lack of reversibility, antithrombotic aptamers can be regulated by antidote oligonucleotides. By binding the aptamer with Watson-Crick base pairing, the antidote oligonucleotide prevents the aptamer from inhibiting its target protein. In preclinical studies, an antidote oligonucleotide for an aptamer inhibiting coagulation factor IXa (FIXa) showed rapid and complete reversal in less than 5 min after administration.[13,14] With the ability to reverse activity in the setting of hemorrhage in addition to unlimited shelf life, and low to no immunogenicity, aptamers show promise to treat thrombosis.[14]

E. Project Overview

As previously described, VWF is a novel and attractive target to exploit for anti-thrombotic therapeutics as it plays a pivotal role in platelet adhesion, activation and aggregation. Our laboratory has developed an RNA aptamer therapeutic (DTRI-031) which binds VWF and inhibits its molecular interaction with the platelet-expressed glycoprotein Ib-IX-V surface receptor, preventing platelet adhesion and aggregation.[6,9] In addition, a matched antidote was developed (**Figure 3**) allowing precise, rapid, and complete reversal of the aptamer *in vivo*, an element not available in current anti-platelet therapies.[9]

rTPA has improved morbidity and mortality in acute ischemic stroke, but hemorrhage risk decreases treatment time to <4.5 hours after symptom onset. In the setting of large vessel occlusion stroke, where patients undergo endovascular thrombectomy, restenosis and thrombosis attributed to subendothelial injury and exposure of VWF during current mechanical thrombectomy therapy results in significant morbidity and mortality.[3,4] Targeting VWF and inhibiting its interaction with GP Ib-IX-V would not only prevent thrombus initiation and platelet aggregation, but can also recanalize a stabilized clot. In addition, the matched aptamer antidote provides an invaluable tool to limit potentially catastrophic intracranial hemorrhage and expand the current treatment window. By utilizing a clinically-relevant stroke model of middle cerebral

arterial occlusion (MCAO), we aimed to determine the thrombolytic efficacy of DTRI-031 in small and large animals by comparing cerebral reperfusion, resulting hemorrhage and stroke volumes to the established rTPA protocol. Consequently, we aimed to demonstrate that DTRI-031 can lyse stabilized clots in a safer and more effective fashion than rTPA. Furthermore, the knowledge of the impact of antithrombotic aptamers may lead to the potential replacement of the current stroke therapeutic paradigm with DTRI-031 for all patients who present with stroke symptoms at any time after symptom onset.

III. MATERIALS AND METHODS

Animals

All experiments were approved by The Ohio State University Institutional Animal Care and Use Committee. Wild-type (WT) C57/BL6 adult mice (8-16 weeks of age) and adult canine beagles (>1 year old) were obtained from Jackson Laboratory and Covance respectively. Mice (n=30; 5 per group male & female) were group housed in a 12:12 light/dark cycle on ventilated racks in a temperature and humidity-controlled vivarium with filtered tap water and *ad libitum* access to food (standard chow). Beagles (n=12; 4 per group), were group housed in a temperature and humidity-controlled vivarium at Wiseman Hall and maintained by ULAR staff.

Middle Cerebral Arterial Occlusion

Acute ischemic stroke was induced by thrombin activation of an autologous clot.[15,16] Mice were anesthetized using a mixture of 55 mg/kg ketamine and 15 mg/kg xylazine. Body temperature was maintained at 37 C using a thermo-regulated heating pad and baseline vital sign, blood pressure (CODA) and heart rate (LabChart) were recorded. An incision in the left saphenous vein and insertion of an intravascular catheter allowed for treatment delivery. An intracranial catheter was inserted into the middle cerebral artery (MCA) containing a mixture of 1

uL of purified mouse thrombin, 13 uL of sterile saline, and autologous blood. Stabilized for 15 minutes, the clot was introduced into the MCA. After 20 minutes, treatment was administered using either DTRI-031 (50 uL bolus over 5 min followed by vehicle (PBB+) at 2 ul/min for 40 min), TPA (10 mg/kg or 2 mg/kg at 2ul/min infused over 45 min), or vehicle (PBB+ at 2 ul/min for 45 min) (**Figure 4**). Blood pressure and heart rate were monitored at perfusion start/stop and at sacrifice. At 24 hours following MCAO, mice were sacrificed, and tissues were harvested for histopathologic evaluation.

Endovascular basilar artery occlusion (BAO) materials and methods were optimized in beagle canines.[15] Preliminary data collected on this procedure has indicated that canine vessel anatomy complicates MCA occlusion. As a result, the basilar artery (BA) was occluded by inserting an intracranial catheter through the femoral artery and introducing an autologous clot. On the day of surgery, beagles were sedated with telazol and anesthetized (isoflurane) and ventilated. Body temperature, end-tidal CO2 and continuous cardiac rhythm monitoring were recorded and maintained within physiologic range. A 1 cm^2 cranial window above the cerebellum was created for laser speckle imaging and an autologous clot was produced by mixing 0.5 g Barium Sulfate and 5 mL autologous whole blood. Bilateral common femoral artery entrance was achieved percutaneously by utilizing 5-French sheaths.[15] With fluoroscopic guidance (GE Medical OEC 9800 Plus Cardiac) a guide catheter, connected to pressurized normal saline, was advanced into (1) vertebral artery.[15] Following this, arteriographic evaluation of the vertebrobasilar arterial system was performed by placing a 4-French catheter through the sheath at the contralateral puncture site during clot deployment. A microcatheter (Boston Scientific, SL-10), through the vertebral artery guide catheter, was then guided by a microwire (Boston Scientific, synchro2 soft) and progressed and tracked into the basilar artery.[15] After the microcatheter was in place, it was manipulated to deliver a 2 mL autologous clot to occlude the BA. Successful BA occlusion was determined angiographically and the total occlusion time was 60 minutes. At 16 frames per second, arteriograms were acquired to evaluate the

vertebrobasilar system using 4 mL aliquots of iohexol (Omnipaque 300, GE Healthcare) injected through the 4-French catheter at the time of occlusion and immediately before drug administration to confirm occlusion.[15] After clot stabilization, treatment was administered intravenously over 45 minutes using either DTRI-031 (0.5 mg/kg), TPA (0.9 mg/kg), or PBB+ (2 mL/min). At 3 hours following occlusion, arteriograms were acquired to evaluate extent of reperfusion. Beagles were then sacrificed, and necropsy was performed to isolate the brain for histopathologic evaluation (**Figure 5-6**).

Visualization of Thrombus In Vivo

Acute ischemic stroke is the result of a sudden occlusion in an arterial vessel which restricts cerebral blood flow and delivery of nutrients including glucose and oxygen to brain tissue. To model this phenomenon in vivo, we employed an autologous clot by inserting a catheter into the basilar artery of beagles. Digital subtraction angiography was performed on beagles to verify successful basilar artery occlusion and reperfusion. Using a long flexible catheter to steer through the blood vessels, a contrast agent (Omnipaque 300, GE Healthcare) was delivered into the arteries, making them visible on the x-ray monitor. A fluoroscope machine (GE OEC 9800 Plus) was utilized to perform this procedure by generating x-rays from one side and photographing them on the other side. The contrast used allows the blood vessels to be visible on the monitor, creating a roadmap of the arteries of interest. Digital subtraction angiography was performed at baseline, after injection of clot to validate successful occlusion, 60 minutes after bolus stop, and at sacrifice.

Measurement of Perfusion and Vasculature

To determine the extent of cerebral flow in beagles, laser speckle imaging was performed at baseline, during injection of clot, throughout treatment, and at sacrifice. Due to the limited depth of the laser, a 1 cm^2 cranial window above the cerebellum was created to improve

the contrast analysis in the region of interest. By generating patterns of reflected light from within the cranial window, the laser speckle imaging device is able to differentiate between stationary and moving particles. With an integrated algorithm using perfusion units (pu) to measure blood flow, stationary particles were calculated around 0 pu and were shown as blue while moving particles were calculated around 200 pu and were shown as red.

Quantification of Stroke and Hemorrhage Volumes

At time of sacrifice, T2 weighted Magnetic Resonance Imaging (MRI) was performed on both mice and beagles to access infarction volumes and downstream thromboembolic events. A Philips 3T MRI scanner (Philips Ingenia CX 3T MRI, Best, Netherlands) was used to acquire MR images for canines using the "Knee coil" (Philips dStream 16 channel Knee coil) as a receiver coil with SENSE enhanced parallel imaging performance. Beagles were anesthetized using isoflurane and positioned head-first supine. MRI scanning parameters used were: T1W: FOV 130 mm, Matrix size 320 x 320, Slice thickness =3 mm, TR= 500 ms, FA= 150 degrees, BW =255 Hz/pixel, NEX= 1, TE=22ms, Resolution= 2.4615 pixels per mm. T2W-TSE: FOV 130 mm, Matrix size 320 x 320, Slice thickness =3 mm, TR= 4000 ms, FA= 180 degrees, BW =255 Hz/pixel, NEX= 2, TE=75ms, Resolution= 2.4615 pixels per mm; T2W-FLAIR: FOV 130 mm, Matrix size 320 x 320, Slice thickness =3 mm, TR= 4000 ms, FA= 180 degrees, BW =255 Hz/pixel, NEX= 2, TE=75ms, Resolution= 2.4615 pixels per mm; DWI: FOV 149x149 mm, Matrix size 132 x0x0x 100, Slice thickness =4 mm, TR= 4600 ms, FA= 90 degrees, BW =255 Hz/pixel, NEX= 1, TE=86ms, Resolution= 0.9333 pixels per mm; DICOM images were transferred for post-processing and OsiriX MD v.5.0 software was used to post process the DICOM series. Two sets of images with b = 0 and 1800 s/mm^2 was used to calculate ADC maps. In addition, coronal sections of the cerebrum in the canine brain were stained with triphenyl-2,3,5-tetrazolium-chloride (TTC) as well as hematoxylin and eosin (H&E) to verify percent reperfusion and infarct volumes. Reaction in metabolically active cells turn red when

placed in TTC, whereas dead cells will not display color. Sections stained with TTC were scanned with a high-resolution scanner and infarct areas (white) were compared with the region of infarct in MRI. Hematoxylin and Eosin (H&E) staining demonstrates live versus dead cells and was also compared to TTC & MRI infarct areas and microembolism. Sections stained with H&E were scanned with a high-resolution slide scanner.

Data Analysis

The results of the experiments were analyzed by several statistical methods using standard software programs (EXCEL, GraphPad Prism) and expressed as mean ± standard deviation. For comparison between two groups, significance will be determined by paired or unpaired Student *t* test. For comparison of multiple groups, multifactorial ANOVA with post hoc comparison will determine significance. For all data analysis, probability values of $p < 0.05$ were considered significant. See table 1 for all experimental outcomes.

IV. RESULTS

Aptamer DTRI-031 significantly improves stroke outcome in a murine model of middle cerebral arterial occlusion

To assess its effectiveness as a thrombolytic agent, we administered DTRI-031 in a murine middle cerebral arterial occlusion stroke model. The primary endpoint of this animal model is to evaluate the thrombolytic activity of DTRI-031 and compare it to rTPA, the most widely used thrombolytic drug and the mainstay of therapy in acute ischemic stroke. After intubation and sedation, the left saphenous vein was exposed and catheterized for treatment delivery. An intracranial catheter was then inserted into the middle cerebral artery (MCA) containing a mixture of 1 uL of purified mouse thrombin, 13 uL of sterile saline, and autologous blood to generate a thrombus. Following 15 minutes of stabilization, the clot was introduced into

the MCA. Once total occlusion had been verified by cessation of blood flow, the occlusive thrombus was left to stabilize for an additional 20 minutes and then aptamer DTRI-031, rTPA, or vehicle (PBB+) was administered. In this experiment, rTPA was dosed at 10 mg/kg which is 11-fold higher than 0.9 mg/kg (the clinical dose administered to human patients who present with ischemic strokes <4.5 hours from symptom onset) because this was the dose reported to be effective for recanalization in murine stroke models. Aptamer DTRI-031 was dosed at 0.5 mg/kg. Following thromboembolic stroke, significant ischemic stroke volumes involving the MCA territory were identified in 81.4% of control-treated animals and in 14.8% of animals treated with DTRI-031 ($p<0.05$) (n=10 per group) (**Figure 7**). Ischemic stroke volumes were significantly higher in animals treated with saline control (7.829 ± 1.988 mm^3) and rTPA (5.362 ± 1.441 mm^3) compared to animals treated with DTRI-031 (1.557 ± 0.5131 mm^3) ($p<0.05$) (n=10 per group) (**Figure 7**). There was no hemorrhagic conversion identified in either cohort.

Aptamer DTRI-031 demonstrates greater cerebral reperfusion in a canine model of thromboembolic stroke compared to rTPA

A canine model of cerebrovascular thrombotic disease was used to corroborate the murine results in a large, clinically relevant animal. Adult beagles were intubated and sedated. The right cephalic artery was catheterized for drug administration and the right femoral artery was catheterized for clot delivery. A 1 cm^2 cranial window above the cerebellum was created for laser speckle imaging and a microcatheter was inserted into the basilar artery for clot delivery. An autologous clot was created by mixing 0.5 g Barium Sulfate and 5 mL autologous whole blood. Arterial occlusion was indicated by laser speckle imaging and verified by digital subtraction angiography of the basilar artery (**Figures 8**). After 60 minutes of occlusion, animals received one of 3 agents: 1) Intravenous injection of 0.5 mg/kg DTRI-031 as a bolus, 2) Intravenous injection of 0.9 mg/kg of rTPA by the standard clinical protocol of 10% injection followed by 90% infusion over 45 minutes, and 3) Intravenous injection of vehicle (PBB+) at 2

mL/min over 45 minutes. No significant difference in baseline cerebellar perfusion was measured between treatment groups (n=4 per group) using laser speckle imaging (LSI). However, induction of BAO injury caused a 58.7 ± 20.2%, 68.7 ± 22.5%, and 70.0 ± 15.5% decrease in perfusion in vehicle, rTPA, and DTRI-031, respectively. DTRI-031 treated animals resulted in 50.0 ± 32.3% of baseline perfusion restored, compared to rTPA with 38.9 ± 38.6% at 3 hours after BAO (**Figure 9**). Images assessed by LSI further revealed 0.5 mg/kg DTRI-031 resulted in a greater percentage of baseline flow restored after injury compared to 0.9 mg/kg rTPA (**Figure 10**). Visualization of perfusion through digital subtraction angiography verified canines treated with DTRI-031 resulted in greater reperfusion before sacrifice compared to rTPA or vehicle (**Figures 11A—C**).

Aptamer DTRI-031 demonstrated reduced infarct volume and no brain hemorrhage or embolization in canines

To evaluate the safety and efficacy of DTRI-031, T2-weighted Magnetic Resonance Imaging (MRI) was performed at time of sacrifice to compare infarction volumes and downstream thromboembolic events to rTPA and vehicle control. In addition, a coronal section of the cerebrum was stained with triphenyl-2,3,5-tetrazolium-chloride (TTC) as well as haematoxylin and eosin (H&E) to verify percent reperfusion and infarct volumes. Dogs were anesthetized using isoflurane and positioned head-first supine. A Philips 3T MRI scanner (Philips Ingenia CX 3T MRI, Best, Netherlands) was used to acquire MR images using the "Knee coil" as a receiver coil with SENSE enhanced parallel imaging performance. DICOM images were transferred for post-processing and OsiriX MD v.5.0 software was used to post process the DICOM series. Two sets of images with b=0 and 1800 s/mm^2 were used to calculate ADC maps. Stroke regions were traced with a red dotted line that matched the stroke region on histological (TTC stained) images (**Figure 12B**). Although the preliminary data collected has provided results for only one canine brain (treated with rTPA, n=1), rTPA

administration induced an infarction volume of 59.91 mL while leaving only 65.67 mL of normal brain tissue. Infarct percentage was calculated at 61.51% for rTPA administration in adult beagle canines (n=1) (**Figure 12A—B**). Histological comparison of treatments (**Figure 13A—F**) confirmed smaller infarction with DTRI-031 compared to rTPA and vehicle. Neither DTRI-031, nor rTPA or vehicle resulted in intracranial hemorrhage or thromboembolic events at time of sacrifice (3 hours after injury) by H&E staining (**Figure 13D—F**). Furthermore, post-reperfusion arteriograms (**Figure 11B**) confirmed reperfusion with DTRI-031 administration with no evidence for peripheral thrombotic events. rTPA and vehicle resulted in no reperfusion before sacrifice (**Figure 11B, C**).

Aptamer DTRI-031 demonstrated no deleterious effects on animal physiology

Mice and beagle vital signs (blood pressure and heart rate) were recorded to assure normal physiological conditions (cardiac function) during the entire surgical procedure and treatment administration. No significant difference was found in mean blood pressure or heart rate between treatment groups (DTRI-031, rTPA, or vehicle) in mice (**Figures 14A—B**) and in beagles (**Figure 15A—D**). All treatment groups in both mice and beagles resulted in normal physiological conditions during the entire surgical procedure.

V. DISCUSSION

The motivation for ischemic stroke research is driven by the fact that current thrombolytic therapy treats only 6% of the over 690,000 stroke patients, while endovascular thrombectomy treats another 15% of patients. The majority of those who suffer ischemic strokes receive no acute therapy. Although rTPA represented a breakthrough, reducing morbidity and improving functional outcomes, rTPA cannot be reversed, and at 6.4%, the risk of hemorrhagic conversion is significant.[2,4] Second, restenosis and occlusion can occur after initial thrombolysis, reversing

initial neurological improvement. Finally, a narrow temporal window to administer rTPA excludes a significant fraction of patients who suffer ischemic cerebrovascular insults (~90%).[4] Several additional stroke therapeutics have been investigated in the past 20 years and while they have shown promise in pre-clinical testing, only a few have been effective in a clinical setting.[25] Due to this urgent need for therapeutic options to treat ischemic stroke patients, we set about testing the efficacy of inhibiting VWF using an RNA aptamer (DTRI-031) against ischemic stroke.

VWF plays an important role in not only platelet adhesion at a site of endothelial injury or atheromatous plaque, but also assists in platelet aggregation and cross-linking through its association with GP IIb/IIIa receptors.[10] Elevated VWF levels are known to place patients at a higher risk of ischemic stroke[23], and VWF inhibition has been shown to ameliorate stroke severity following transient MCAO in pre-clinical models.[26,27] Given the central role of VWF in thrombus initiation and propagation, it represents an attractive target for both thrombolysis and prevention of secondary thrombosis and re-occlusion.

In previous studies, DTRI-031 inhibition of VWF has demonstrated efficacy as a potent anti-platelet agent with the ability to prevent thrombus formation *in vivo*.[8] In this study we aimed to investigate the thrombolytic efficacy of DTRI-031 by utilizing clinically relevant stroke models with small and large animals. In the murine middle cerebral artery occlusion model, we demonstrated a significant decrease in stroke volume in DTRI-031 treated mice compared to rTPA and control (**Figure 7**). No deleterious effects on mice physiological conditions were recorded during DTRI-031 treatment (**Figure 14**). We then aimed to corroborate the murine results in a large, clinically relevant animal. Using a canine model of cerebrovascular thrombotic disease, we occluded the basilar artery in beagles and investigated the thrombolytic efficacy of DTRI-031 by comparing it to rTPA and control. We found that Inhibition of VWF by DTRI-031 resulted in greater restoration of perfusion compared to rTPA and control at 60 minutes after placement of an autologous clot (**Figures 9-11**). Additionally, DTRI-031 treated canines showed less infarction with no evidence of thromboembolism, results reflected in our murine MCAO

experiments (**Figure 13).** No deleterious effects on beagle physiological conditions were recorded during DTRI-031 treatment (**Figure 15**).

The preliminary results presented in this study demonstrate that DTRI-031 inhibition of VWF is an effective anti-thrombotic agent in both small and large animal models of thromboembolic stroke. With further investigation and data analysis, we believe VWF inhibition by DTRI-031 may represent a replacement for rTPA therapy for the >90% of stroke patients who are left with little thrombolytic options. Moreover, we believe that the antidote-controlled approach we have developed, taken together with the highly potent antithrombotic and thrombolytic efficacy of the VWF aptamer DTRI-031, could provide an effective and safer treatment option not only in acute ischemic stroke, but may be generalizable to coronary and peripheral artery disease as well.

Table 1

Experimental Outcomes	Murine	Canine
Magnetic Resonance Imaging (stroke and hemorrhage volumes)	✓	✓
Angiography (extent of occlusion and reperfusion)		✓
Speckle (blood flow velocity throughout the brain)		✓
Histology (H&E staining and TTC staining to verify MRI)	✓	✓
Physiological Effect (Verify physiological surgery conditions including ECG, blood gas analysis, CBC, HR, BP)	✓	✓

VI. FIGURES

Figure 1

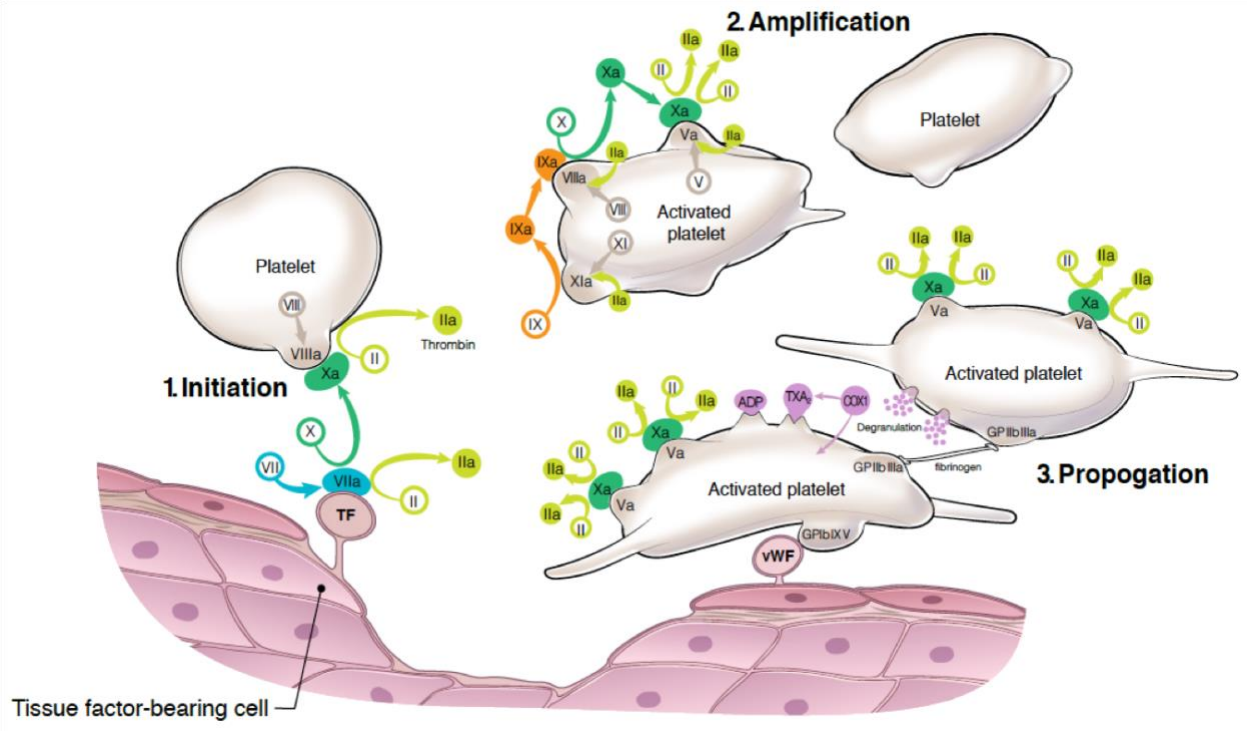

Figure 1: The interaction between Von Willebrand Factor (VWF) and glycoprotein Ib-IX-V (gpIb-IX-V) presents an attractive target for pharmacotherapy.

Figure 2

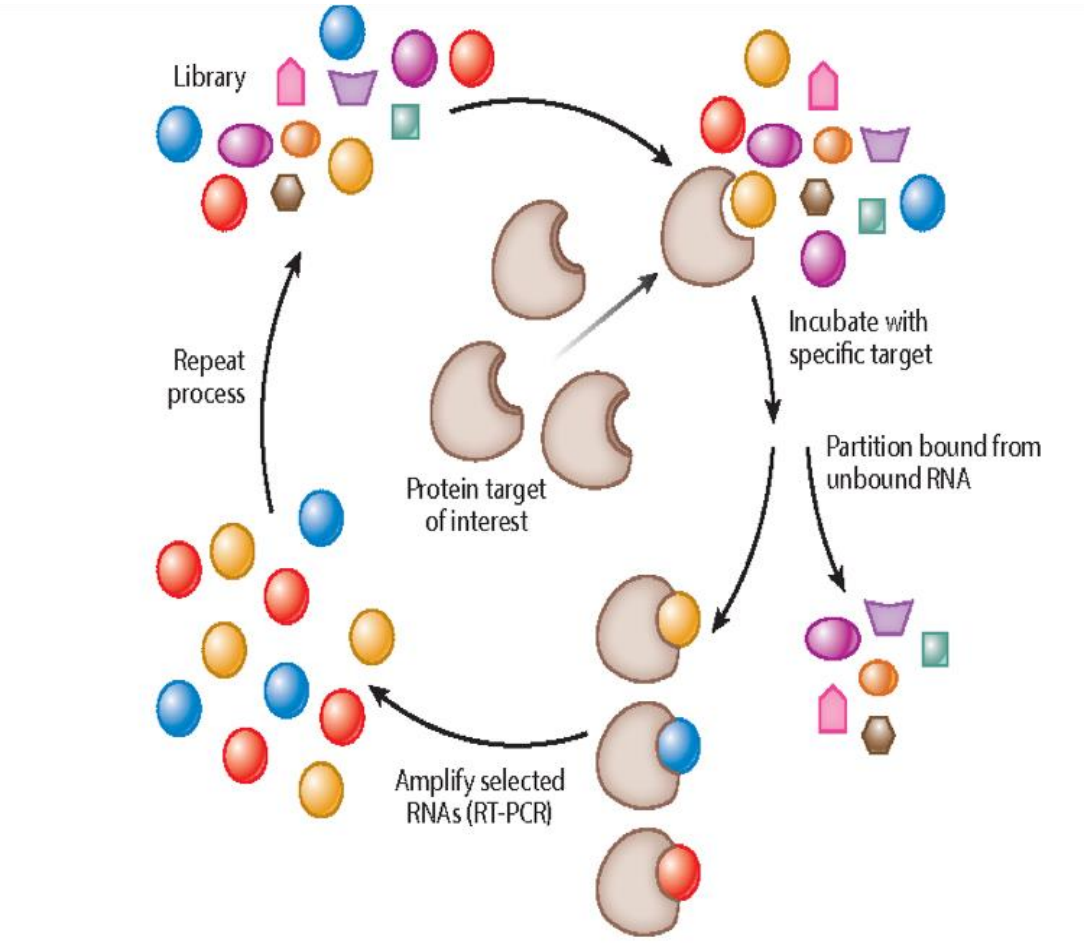

Figure 2: Systematic evolution of ligands by exponential enrichment (SELEX). Technique used in molecular biology for producing oligonucleotides of either single-stranded DNA or RNA that specifically binds to a target ligand, referred to as aptamers.

Figure 3

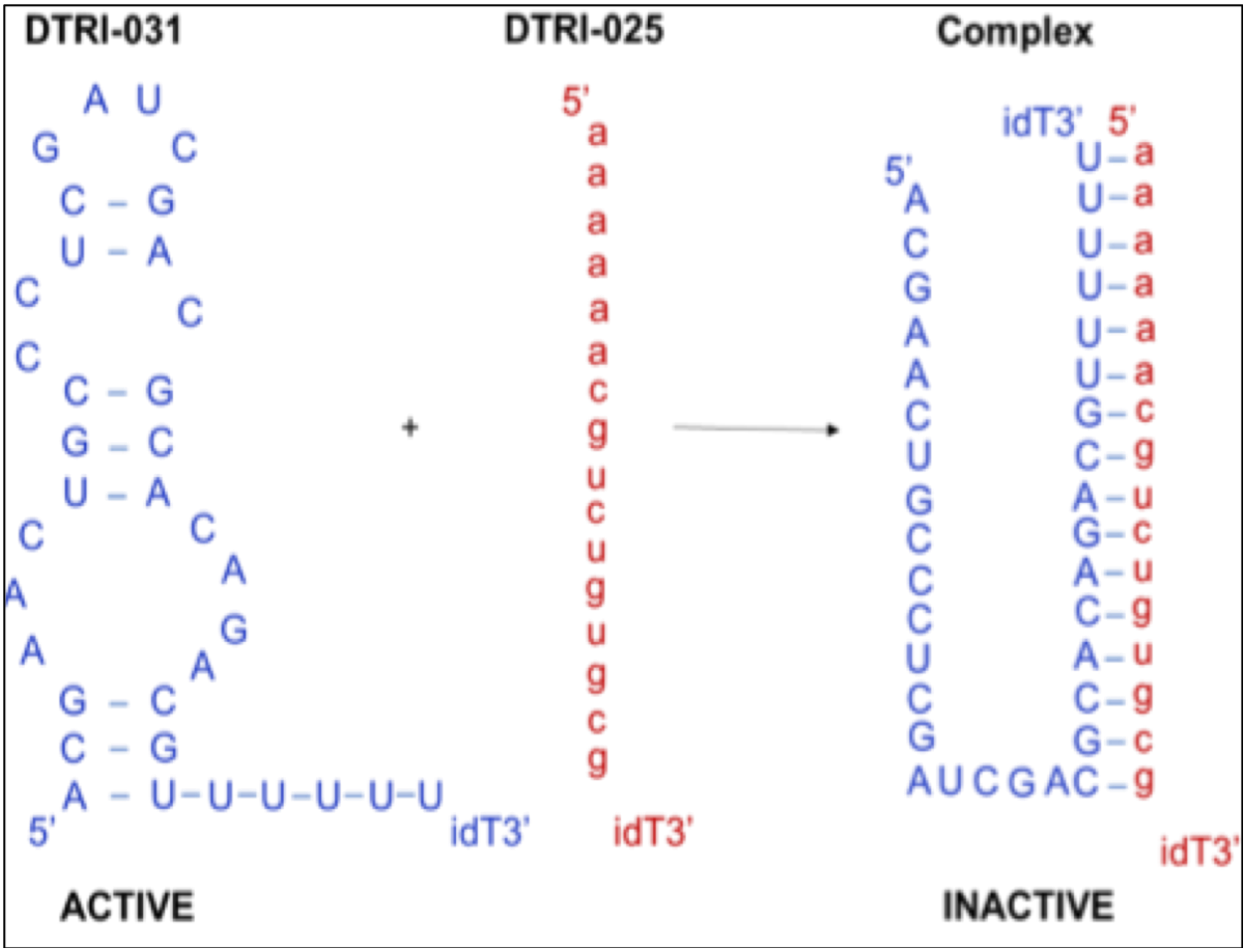

Figure 3: Antidote oligonucleotide (DTRI-025) binds to VWF aptamer (DTRI-031) by Watson Crick base pairing preventing it from binding to the target protein.

Figure 4

Sex	PBB+ (5 min bolus)	Aptamer (0.5 mg/kg, 5 min bolus)	rTPA (10 mg/kg 10% bolus, then 45 min infusion)
Male	5	5	5
Female	5	5	5
Total	**5**	**5**	**5**

Figure 4: Murine treatment groups (n=30). Equal number of male and female mice distributed into different experimental groups

Figure 5

Sex	PBB+ (5 min bolus)	Aptamer (0.5 mg/kg, 5 min bolus)	rTPA (0.9 mg/kg 10% bolus, then 45 min infusion)
Male	2	3	3
Female	2	1	1
Total	**4**	**4**	**4**

Figure 5: Canine treatment groups (n=12). Equal number of canines distributed into different experimental groups.

Figure 6

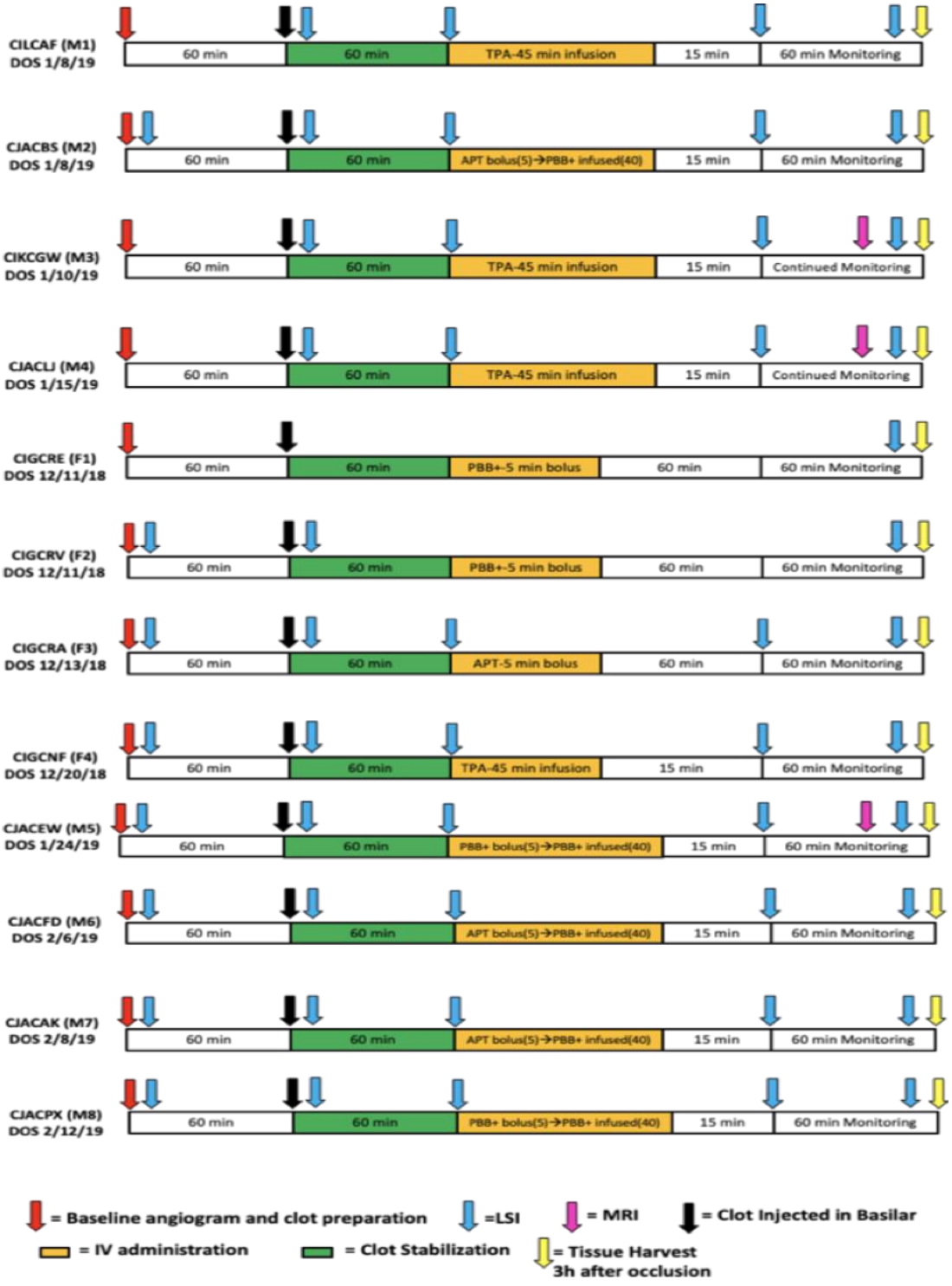

Figure 6: Protocols for in vivo canine experiments. Cerebral flow was recorded and stabilized for a period of 5 min with continuous ECG, heart rate, and blood pressure monitoring throughout. Tissue harvest was collected 3 hours after occlusion.

Figure 7

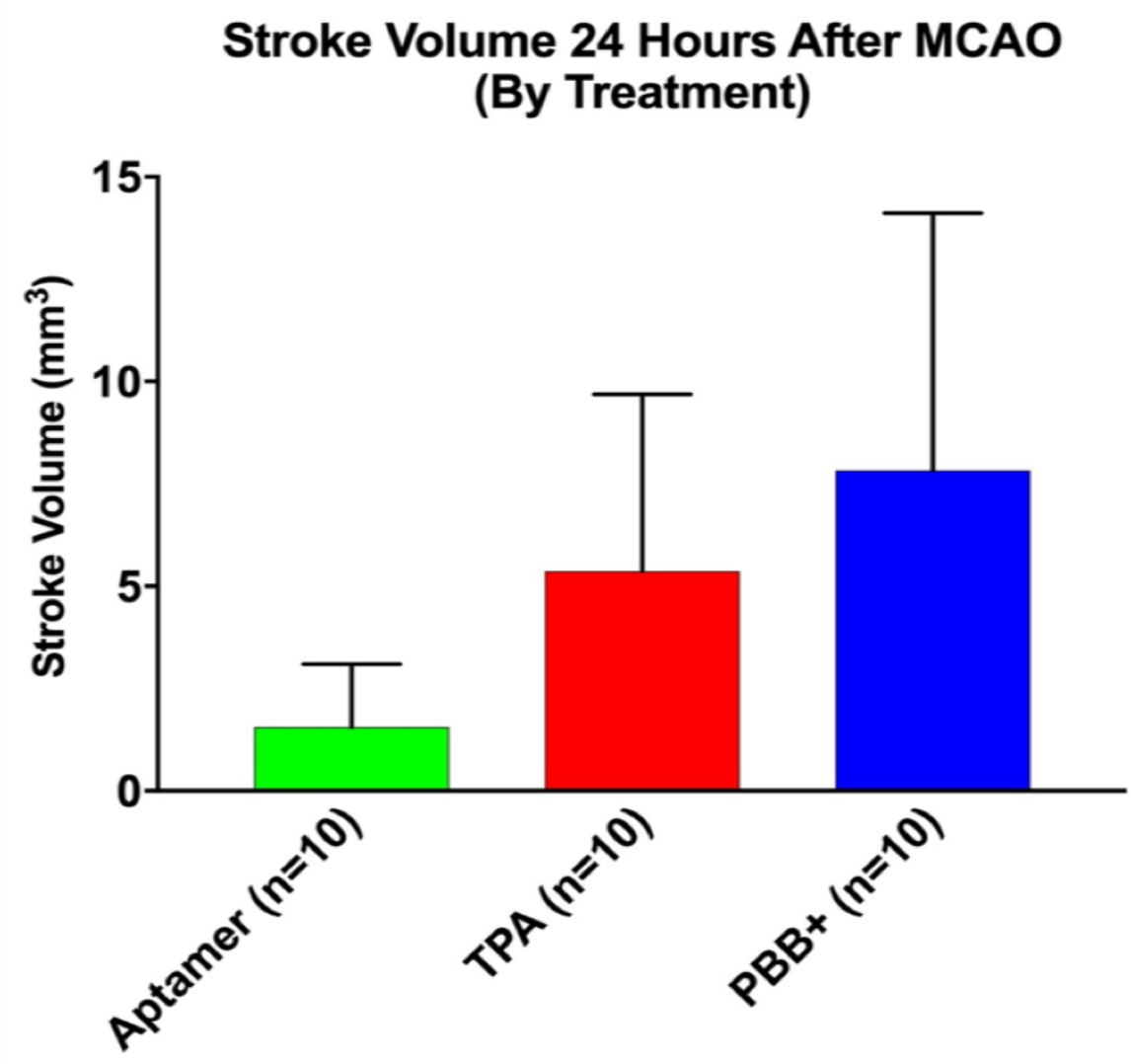

Figure 7: Stroke volume was significantly decreased in mice treated with VWF aptamer (1.557 ± 0.5131 mm^3) compared to rTPA (5.362 ± 1.441 mm^3) and vehicle (7.829 ± 1.988 mm^3). $P<0.05$

Figure 8

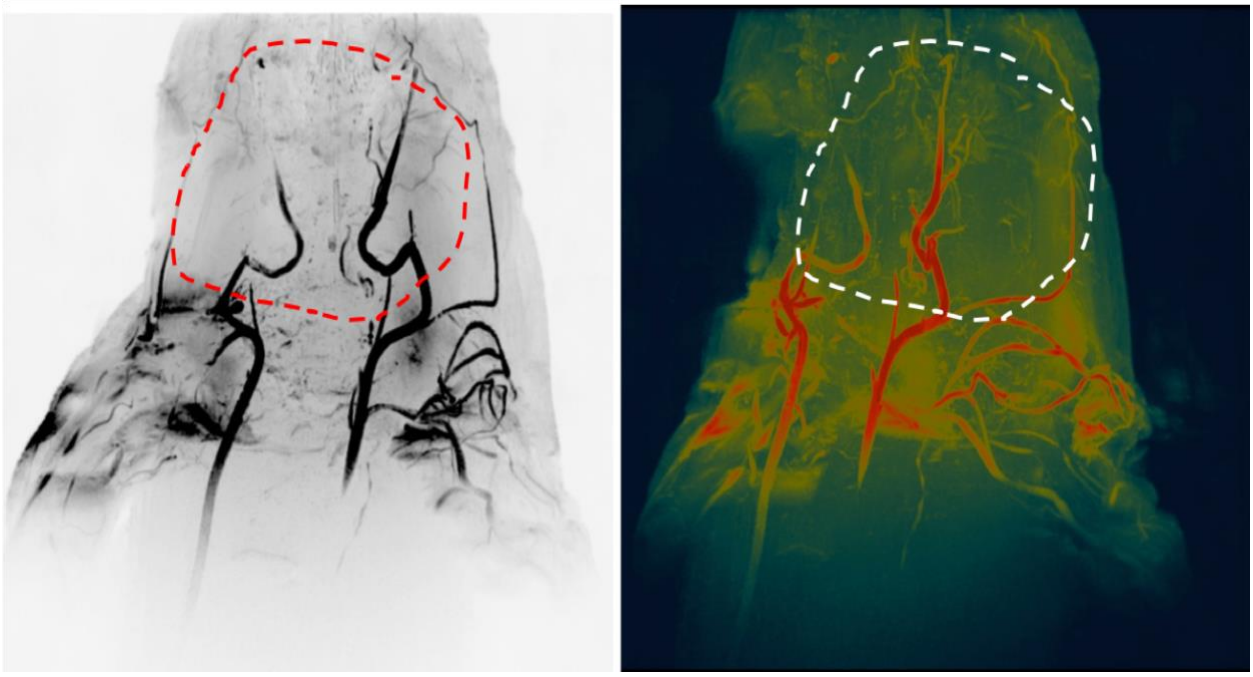

Figure 8: Magnetic Resonance Angiography (MRA) confirmed basilar artery occlusion in beagle canines.

Figure 9

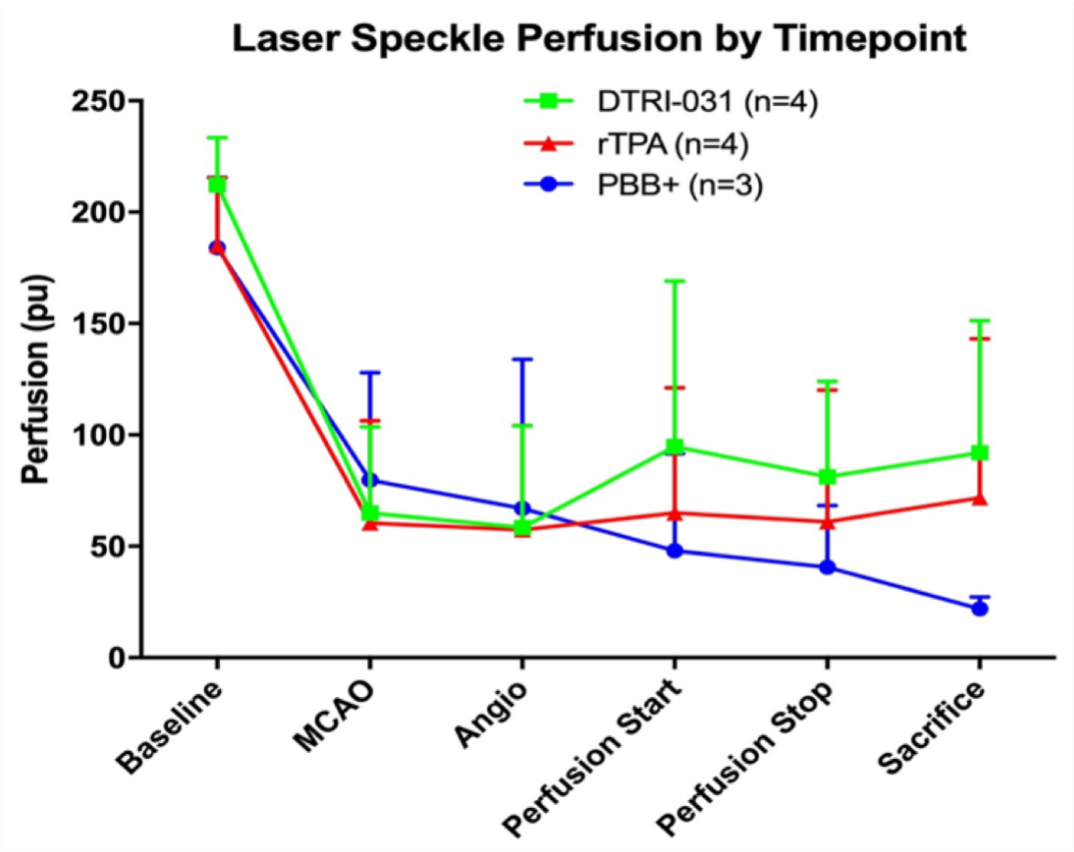

Figure 9: BA perfusion measured by Laser Speckle Imaging (LSI). Beginning with no significant difference in baseline cerebellar perfusion, induction of BAO injury caused a 58.7±20.2%, 68.7±22.5%, and 70.0±15.5% decrease in perfusion in vehicle, rTPA, and DTRI-031, respectively. DTRI-031 resulted in 50.0±32.3% of baseline perfusion restored, compared to rTPA with 38.9±38.6% at 3 hours after BAO.

Figure 10

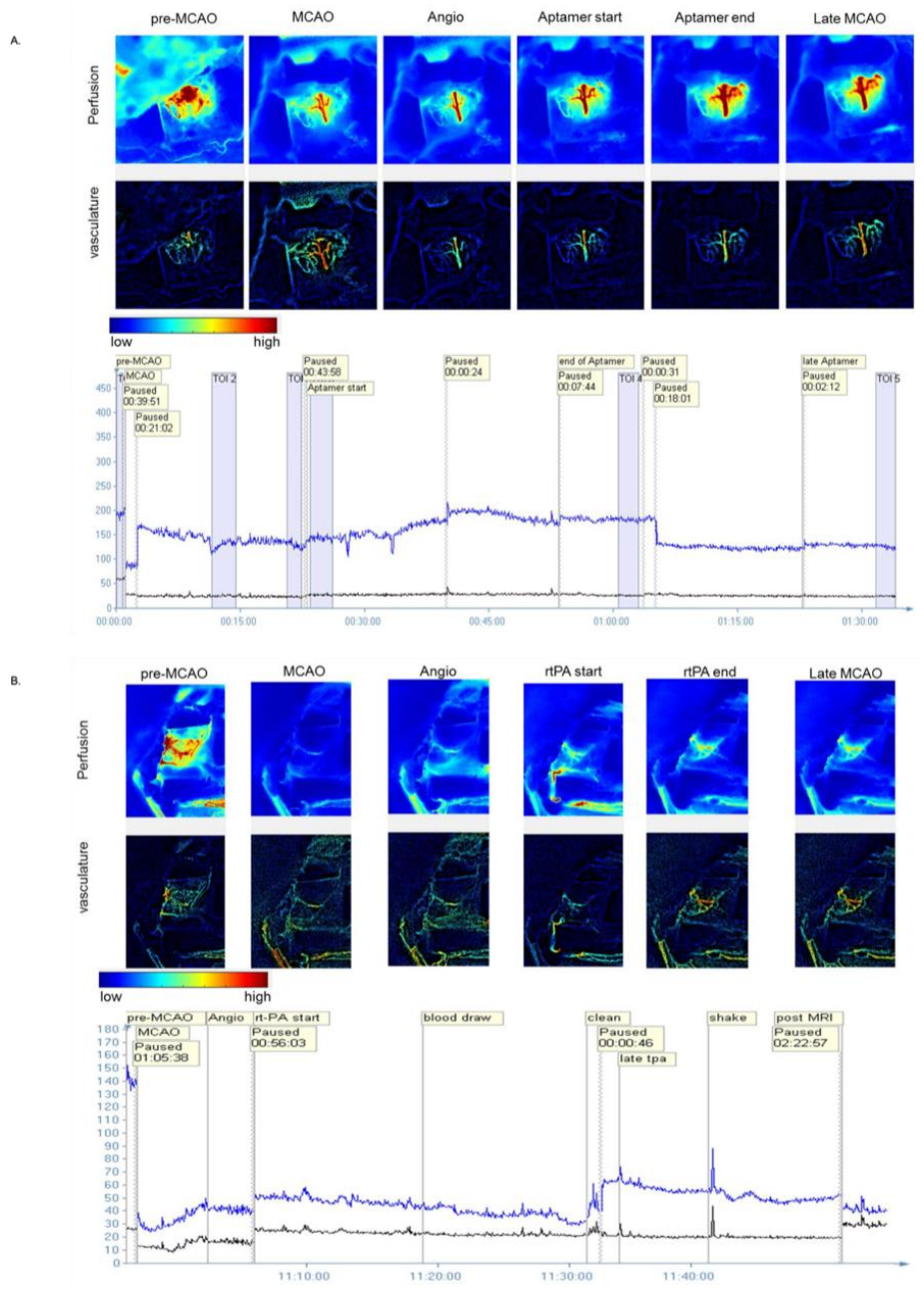

Figure 10: Laser Speckle Imaging (LSI) comparison of DTRI-031 and rTPA treatments. 0.5 mg/kg VWF aptamer resulted in a greater percentage of baseline flow restored 3 hours after injury in Male Dog 7 (CJACAK) (A) compared to 0.9 mg/kg rTPA in Male Dog 4 (CJACLJ) (B) assessed with LSI.

Figure 11

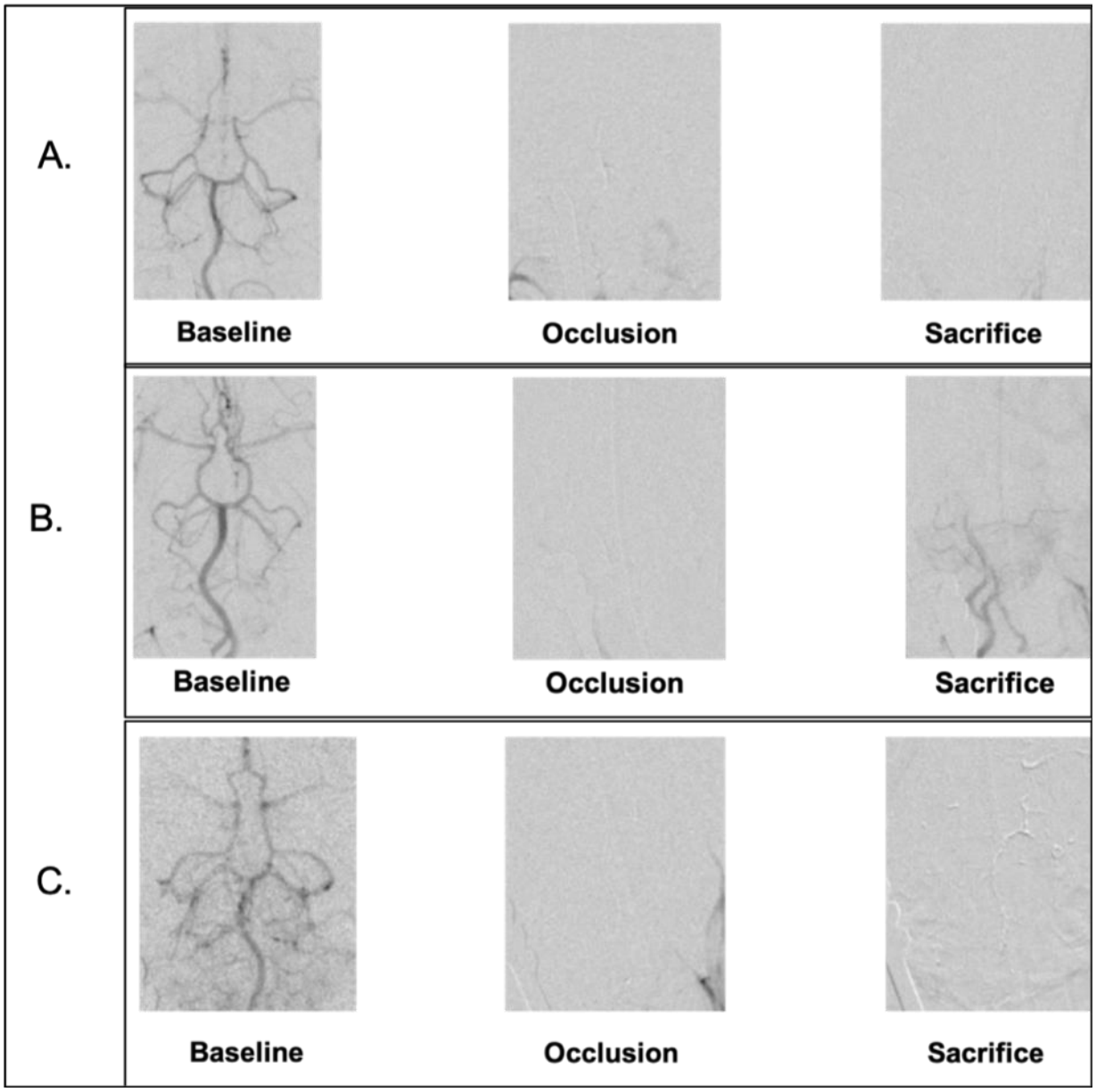

Figure 11: Digital Subtraction Angiography images comparing extent of occlusion and reperfusion between treatments. 0.5 mg/kg VWF aptamer resulted in greater reperfusion at sacrifice in Male Dog 7 (CJACAK) (B) compared to 0.9 mg/kg rTPA in Male Dog 4 (CJACLJ) (A) or saline control in Male Dog 5 (CJACEW) (C) assessed with intra-operative fluoroscopy.

Figure 12

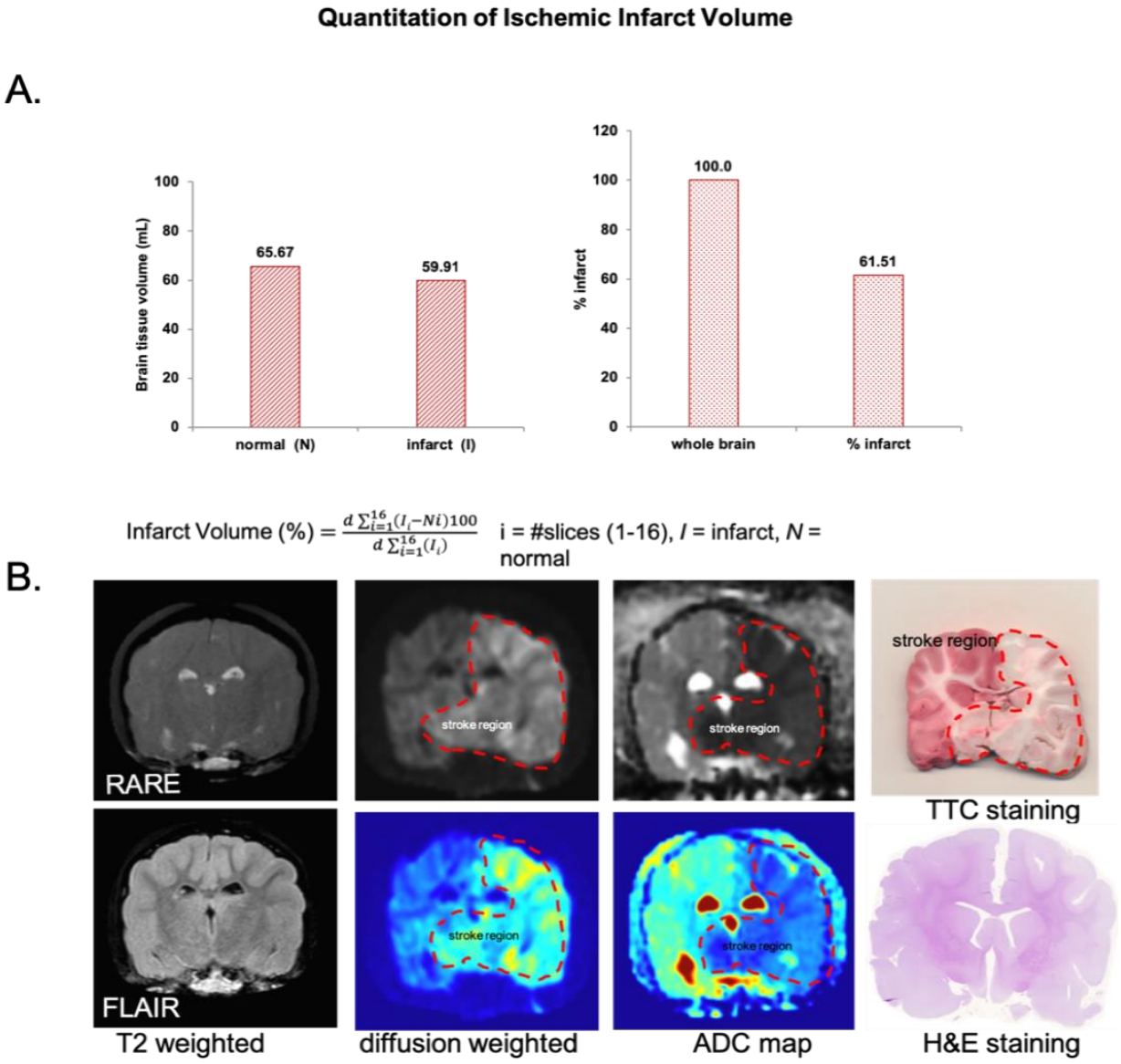

Figure 12: Preliminary data showing quantification of stroke and hemorrhage volume. rTPA administration on Male Dog 4 (CJACLJ) induced an infarction volume of 59.91 mL (61.51% infarct) (A). Representative image of T2-weighted high-resolution magnetic resonance imaging after MCAO was induced in Male Dog 4 (CJACLJ) treated with rTPA (B). A Philips 3T MRI scanner (Philips Ingenia CX 3T MRI, Best, Netherlands) was used to acquire MR images using the “Knee coil” (Philips dStream 16 channel Knee coil) as a receiver coil with SENSE enhanced parallel

imaging performance. Dog was anesthetized using isoflurane and was positioned head-first supine. MRI scanning parameters used were:T1W: FOV 130 mm, Matrix size 320 x 320, Slice thickness =3 mm, TR= 500 ms, FA= 150 degrees, BW =255 Hz/pixel, NEX= 1, TE=22ms, Resolution= 2.4615 pixels per mm. T2W-TSE: FOV 130 mm, Matrix size 320 x 320, Slice thickness =3 mm, TR= 4000 ms, FA= 180 degrees, BW =255 Hz/pixel, NEX= 2, TE=75ms, Resolution= 2.4615 pixels per mm; T2W-FLAIR: FOV 130 mm, Matrix size 320 x 320, Slice thickness =3 mm, TR= 4000 ms, FA= 180 degrees, BW =255 Hz/pixel, NEX= 2, TE=75ms, Resolution= 2.4615 pixels per mm; DWI: FOV 149x149 mm, Matrix size 132 x0x0x 100, Slice thickness =4 mm, TR= 4600 ms, FA= 90 degrees, BW =255 Hz/pixel, NEX= 1, TE=86ms, Resolution= 0.9333 pixels per mm; DICOM images were transferred for post-processing and OsiriX MD v.5.0 software was used to post process the DICOM series. Two sets of images with b = 0 and 1800 s/mm^2 was used to calculate ADC maps. Stroke region is traced with a red dotted line that matches the stroke region on histological (TTC stained) image.

Figure 13

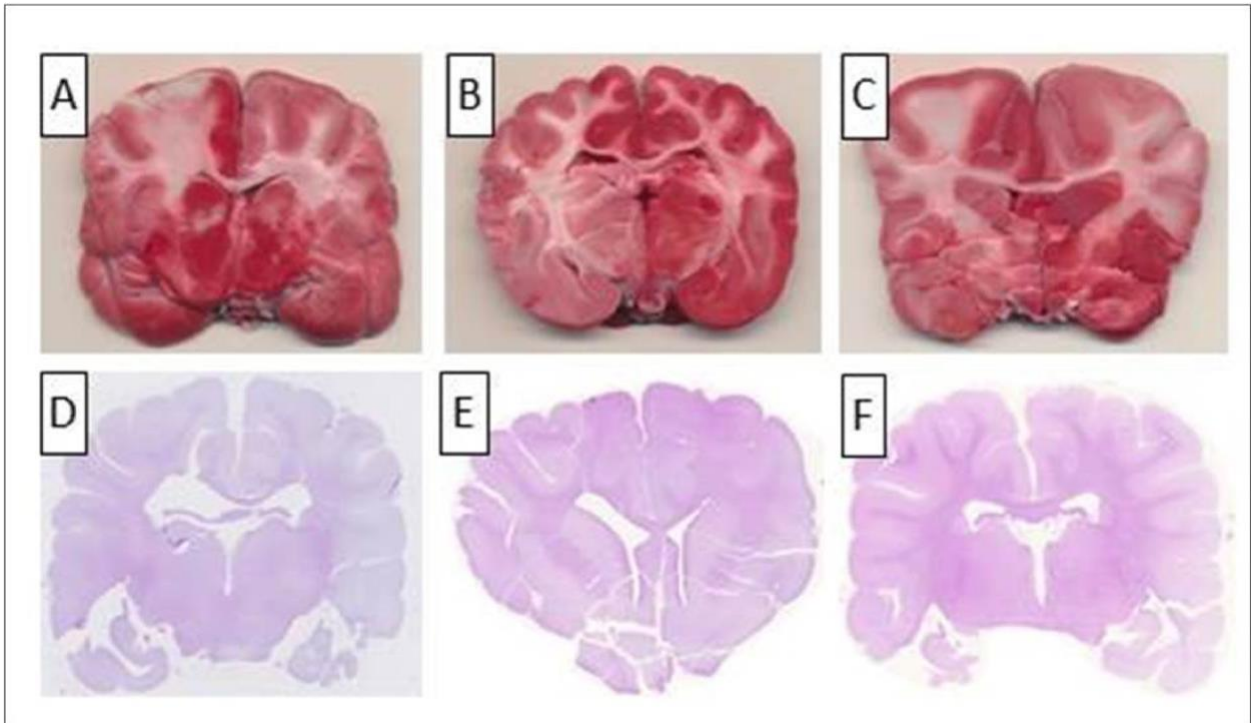

Figure 13: Histological comparison of treatments. TTC staining of canine brain confirmed smaller infarction with DTRI-031 (A) compared to rTPA (B) and vehicle (C). Neither DTRI-031 (D), nor rTPA (E) or vehicle (F) resulted in intracranial hemorrhage or thromboembolic events at time of sacrifice (3 hours after injury).

Figure 14

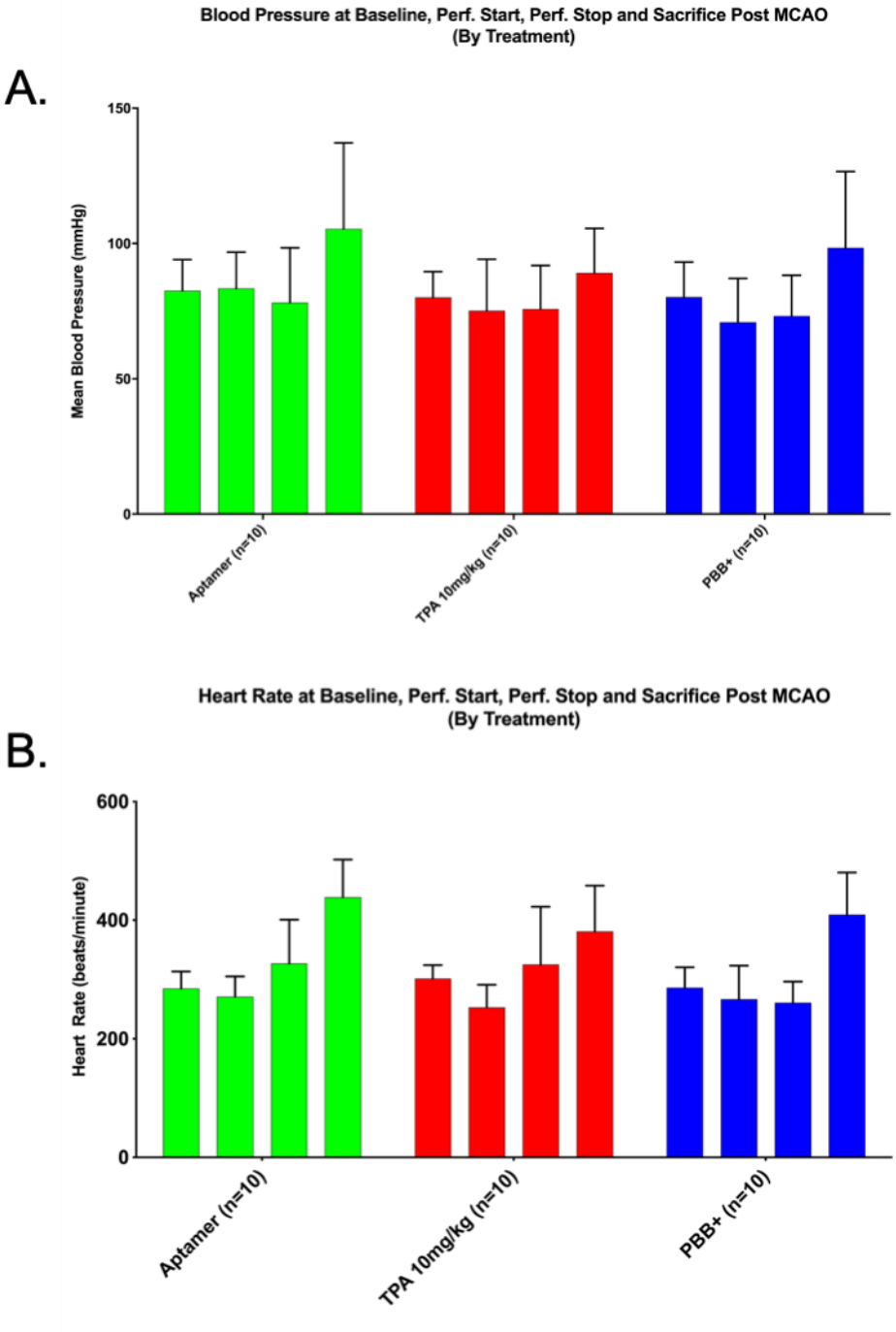

Figure 14: Physiological comparison of treatments in a murine model of thromboembolic stroke. No significant difference in blood pressure (A) or heart rate (B) was recorded between treatments at baseline, perfusion start, perfusion stop and sacrifice.

Figure 15

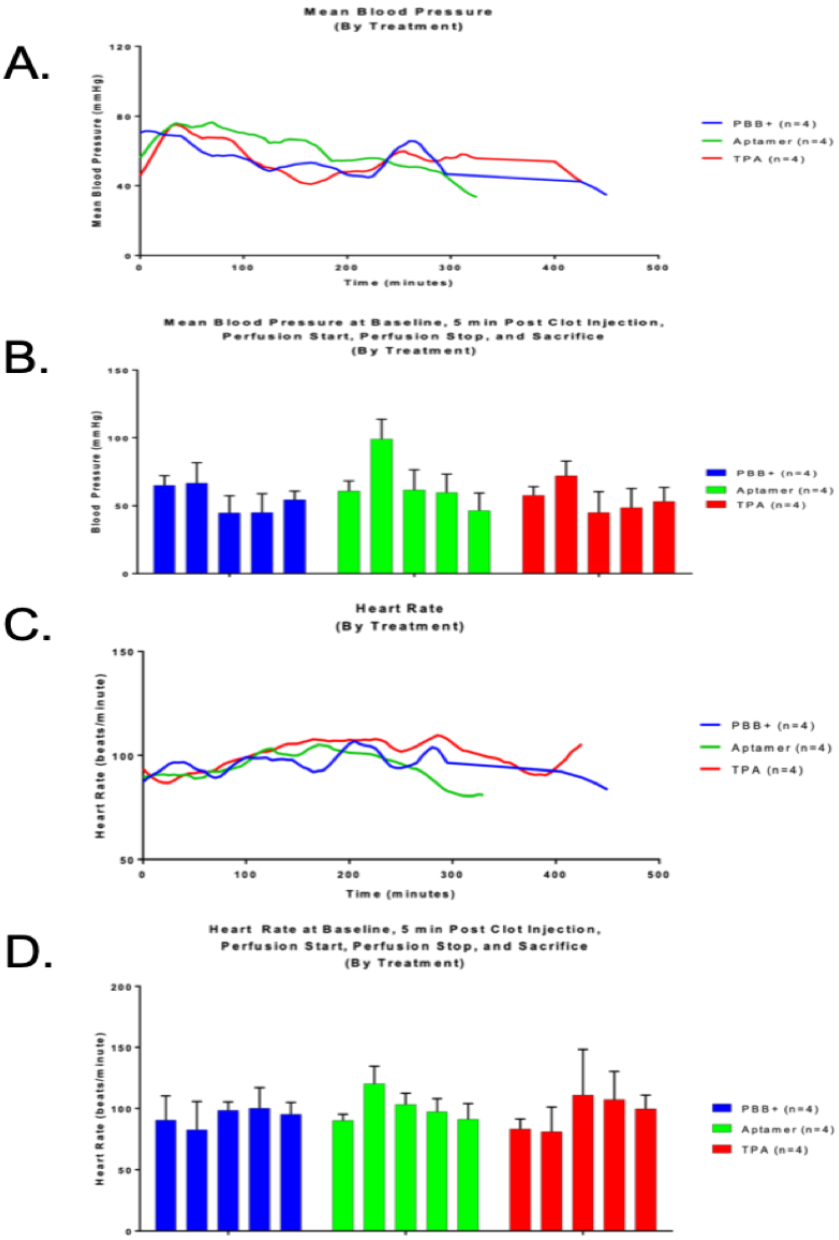

Figure 15: Physiological comparison of treatments in a canine model of thromboembolic stroke. No significant difference was recorded between treatments during continuous blood pressure monitoring (mean blood pressure) (A) or in mean blood pressure recorded (B) at baseline, perfusion start, perfusion stop and sacrifice. Furthermore, no significant difference was recorded during continuous cardiac rhythm monitoring (C) or in heart rate recorded (D) at baseline, perfusion start, perfusion stop and sacrifice.

VII. ACKNOWLEDGEMENTS

I would like to thank Dr. Surya Gnyawali, PhD, and Matthew Joseph, BS for their assistance with experimentation and data analysis. I would also like to thank Dr. Shahid Nimjee, MD, PhD for his support, guidance and critical reading of this manuscript. Moreover, countless thanks go to the entire Nimjee Lab for their mentorship and dedication to science. Finally, I would like to give a special thank you to Ms. Debra Wheeler, BS as this project would not have been possible without your guidance and support.

VIII. REFERENCES

1. "What Is Stroke?" *National Stroke Association*. www.stroke.org/understand-stroke/what-is-stroke/
2. Vernino S, Brown RD, Sejvar JJ, Sicks JD, Petty GW, O'Fallon WM. 2003. Cause-Specific Mortality After First Cerebral Infarction: A Population-Based Study. *Stroke* 34:1828-32
3. National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group.1995. Tissue Plasminogen Activator for Acute Ischemic Stroke. *New England Journal of Medicine* 333:1581-8
4. Adams HP, Jr. 2003. Stroke: a vascular pathology with inadequate management. *J Hypertens Suppl* 21:S3-7
5. Lansberg MG, Bluhmki E, Thijs VN. 2009. Efficacy and safety of tissue plasminogen activator 3 to 4.5 hours after acute ischemic stroke: a metaanalysis. *Stroke* 40:2438-41
6. Nagel S, Schellinger PD, Hartmann M, Juettler E, Huttner HB, et al. 2009. Therapy of acute basilar artery occlusion: intraarterial thrombolysis alone vs bridging therapy. *Stroke* 40:140-6 Freedman JE. 2005. Molecular regulation of platelet-dependent thrombosis. *Circulation* 112:2725-34
7. Nimjee, SM. Coagulation for the Neurosurgeon. *Youmans and Winn Neurological Surgery*. Philadelphia: Elsevier, Inc, pp. 142-8, 2017.
8. Kumar V. Cotran RS, Robbins SL. *Basic Pathology*. 6th ed. Philadelphia: W.B. Saunders; 1997
9. Nimjee SM. Rapidly regulating platelet activity in vivo with an antidote controlled platelet inhibitor. *Molecular therapy*. 02/2012;20(2):391-397. doi: 10.1038/mt.2011.226.
10. Denis CV, Wagner DD. Platelet adhesion receptors and their ligands in mouse models of thrombosis. Arterioscler Thromb Vasc Biol. 2007;27(2):728–739. [PubMed: 17272754]

11. Ciccone A, Motto C, Abraha I, Cozzolino F, Santilli I. 2014. Glycoprotein IIb-IIIa inhibitors for acute ischaemic stroke. *The Cochrane database of systematic reviews* 3:CD005208
12. Adams HP, Jr., Effron MB, Torner J, Davalos A, Frayne J, et al. 2008. Emergency administration of abciximab for treatment of patients with acute ischemic stroke: results of an international phase III trial: Abciximab in Emergency Treatment of Stroke Trial (AbESTT-II). *Stroke* 39:87-99
13. Nimjee SM. Aptamers as Therapeutics. *Annu Rev Pharmacol Toxicol*. 2017;57:61-79. doi:10.1146/annurev-pharmtox-010716-104558
14. Nimjee SM. The potential of aptamers as anticoagulants. *Trends in Cardiovascular Medicine*. 01/2005;15(1):41-45. doi: 10.1016/j.tcm.2005.01.002
15. Christoforidis GA, Rink C, Kontzialis MS, Mohammad Y, Koch RM, Abduljalil AM, Bergdall VK, Roy S, Khanna S, Slivka AP, Knopp MV, Sen CK. 2011. An endovascular canine middle cerebral artery occlusion model for the study of leptomeningeal collateral recruitment. Invest Radiol. 2011 Jan;46(1):34-40.
16. Sheng Liu 2012 - A novel embolic stroke model resembling lacunar infarction following proximal middle cerebral artery occlusion in beagle dogs
17. Conway, DS, Pearce, LA, Chin, BS, HART, RG and Lip, GY (2003). Prognostic value of plasma von Willebrand factor and soluble P-selectin as indices of endothelial damage and platelet activation in 994 patients with nonvalvular atrial fibrillation. *Circulation* **107:** 3141-3145
18. Kamphuisen, PW, Eikenboom, JC and Bertina, RM (2001). Elevated factor VIII levels and the risk of thrombosis. *Arterioscler Thromb Vasc Biol* **21:** 731-738
19. Cosmi, B (2009). ARC-1779, a PEGylated aptamer antagonist of von Willebrand factor for potential use as an anticoagulant or antithrombotic agent. Curr Opin Mol Ther 11: 322–328.

20. Diener, JL, Daniel Lagassé, HA, Duerschmied, D, Merhi, Y, Tanguay, JF, Hutabarat, R et al. (2009). Inhibition of von Willebrand factor-mediated platelet activation and thrombosis by the anti-von Willebrand factor A1-domain aptamer ARC1779. J Thromb Haemost 7: 1155–1162.
21. Wu, D, Vanhoorelbeke, K, Cauwenberghs, N, Meiring, M, Depraetere, H, Kotze, HF et al. (2002). Inhibition of the von Willebrand (VWF)-collagen interaction by an antihuman VWF monoclonal antibody results in abolition of in vivo arterial platelet thrombus formation in baboons. Blood 99: 3623–3628.
22. Yamamoto, H, Vreys, I, Stassen, JM, Yoshimoto, R, Vermylen, J and Hoylaerts, MF (1998). Antagonism of vWF inhibits both injury induced arterial and venous thrombosis in the hamster. Thromb Haemost 79: 202–210.
23. Cadroy, Y, Hanson, SR, Kelly, AB, Marzec, UM, Evatt, BL, Kunicki, TJ et al. (1994). Relative antithrombotic effects of monoclonal antibodies targeting different platelet glycoprotein-adhesive molecule interactions in nonhuman primates. Blood 83: 3218–3224.
24. Oney S, Nimjee SM, Layzer J, Que-Gewirth N, Ginsburg D, et al. 2007. Antidote-controlled platelet inhibition targeting von Willebrand factor with aptamers. *Oligonucleotides* 17:265-74
25. Nimjee, SM, Povsic, T, Sullenger, BA, Becker, RC. Translation and clinical development of antithrombotic aptamers. *Nucleic Acid Therapeutics*. Vol. 26(3): 147-55, June 2016
26. Kidwell, C.S., Liebeskind, D.S., Starkman, S. & Saver, J.L. Trends in acute ischemic stroke trials through the 20th century. Stroke; a journal of cerebral circulation 32, 1349-1359 (2001).
27. De Meyer SF, Schwarz T, Deckmyn H, Denis CV, Nieswandt B, Stoll G, et al: Binding of von Willebrand Factor to Collagen and Glycoprotein Ibα, But Not to Glycoprotein IIb/IIIa,

Contributes to Ischemic Stroke in Mice—Brief Report. Arteriosclerosis, Thrombosis, and Vascular Biology 30:1949-1951, 2010

28. De Meyer SF, Schwarz T, Schatzberg D, Wagner DD: Platelet glycoprotein Ibalpha is an important mediator of ischemic stroke in mice. Exp Transl Stroke Med 3:9, 2011

29. Campbell B, Mitchell P, Churilov L, Yassi N, Kleinig T, Dowling R, et al: Tenecteplase versus Alteplase before Thrombectomy for Ischemic Stroke. *New England Journal of Medicine* 2018; 378:1573-1582.

30. Benjamin E.J, Virani S, Callaway C, et al: Heart Disease and Stroke Statistics: A Report From the American Heart Association. 2018; 137:e67-e492